RESEARCH ARTICLE

Protective Effects of *Scutellaria barbata* Against Rat Liver Tumorigenesis

Zhi-Jun Dai[&*], Wen-Ying Wu[&], Hua-Feng Kang[&], Xiao-Bin Ma[&], Shu-Qun Zhang, Wei-Li Min, Wang-Feng Lu, Shuai Lin, Xi-Jing Wang*

Abstract

   Scutellaria barbata D. Don (S. barbata), a traditional Chinese medicine, is used to treat cancers, inflammation, and urinary diseases. This study aimed to determine any protective effects of *S. barbata* crude extract (CE-SB) against rat liver tumorigenesis induced by diethylnitrosamine (DENA). Liver malfunction indices in serum were measured by biochemical examination. Hematoxylin and eosin staining was performed to examine liver pathology. Contents of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured in liver homogenates to evaluate oxidative stress. The levels of liver malfunction indices in the CE-SB groups, especially in the CE-SB high dose group, were lower than that of the model group ($P<0.05$). The results from histological examination indicated that the number of liver nodules in the CE-SB groups decreased compared with the model group ($P<0.05$). Content of MDA determined in liver was significantly decreased, and level of SOD elevated by CE-SB. CE-SB can inhibit experimental liver tumorigenesis and relieve hepatic injury in rats.

Keywords: Anti-tumor - *Scutellaria barbata* - hepatoma - tumorigenesis - diethylnitrosamine - rat

Introduction

Liver cancer is one of the most common cancers with high incidence and mortality. Curative surgery or liver transplantation is rarely possible and often leads to tumor recurrence (Jemal et al., 2011). Although many therapy strategies for liver cancer exist, the therapeutic outcome remains very poor (Farazi1 et al., 2006). So, the preventive control of liver cancer has been emphasized for the limited liver cancer treatment and grave prognosis. Many Chinese herbs that could be potential antitumor drug sources have been discovered (Vickers et al., 2002). The herb has been used in clinics to treat lung cancer, digestive system cancers, hepatoma, breast cancer, and chorioepithelioma.

Scutellaria barbata D. Don (S. barbata) is a perennial herb, which mainly grows throughout southern China. The S. barbata D. Don herb is known in traditional Chinese medicine as Ban-Zhi-Lian, and it has been used as an anti-inflammatory and antitumor agent as well as a diuretic in China and Korea (Lin et al., 1996; Lee et al., 2004; Yin et al., 2004; Goh et al., 2005; Suh et al., 2007; Dai et al., 2008). S. barbata contains a large number of alkaloids, flavones, sterides, and polysaccharides (Dai et al., 2007; Mi et al., 2010; Qu et al., 2010; Dai et al., 2011). The crude extracts of S. barbata (CE-SB) have in vitro growth inhibitory effects on numerous human cancers including colon cancer, lung cancer, hepatoma, and skin cancer (Yin et al., 2004; Goh et al., 2005; Suh et al., 2007; Dai et al., 2008). Furthermore, our previous research found that CE-SB have antitumor activities both in vitro and in vivo (Dai et al., 2011).

Diethylnitrosamine (DENA) is a well-known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics, and pharmaceutical products (Lee et al., 1998). DENA induces damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models (Bhosale et al., 2002). The DENA-induced disease process is similar to human liver cancer. In the present study, the protective effects of CE-SB against DENA-induced rat liver tumorigenesis were investigated.

Materials and Methods

Reagents and animals

Sixty adult male Sprague–Dawley rats weighing 200 g to 220 g were purchased from the Experiment Animal Center, Medical School of Xi'an Jiaotong University, China. The rats were housed (five per cage) in an air-conditioned room at a constant temperature of 23 °C±1 °C and a humidity of 60±10% with a 12 h light/dark

*Department of Oncology, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China &Equal contributors *For correspondence: dzj0911@126.com, wangxijing@21cn.com*

cycle for 1 week before the experimental period. Food and water were available ad libitum. The experiments were conducted according to Institutional and National Guidelines. DENA, enzymes, and coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CE-SB was purchased from Xi'an zhong-xin Biotechnology Development Ltd. (Xi'an, P.R. China). Other chemicals were AnalaR grade. DENA was prepared as a saline solution with a concentration of 8 mg/mL.

Experimental Protocol

HCCs were induced as described previously (Futakuchi et al., 1999) with slight modifications. The rats were given a single intraperitoneal injection of DENA at a dose of 100 mg/kg body weight to induce liver carcinogenesis, followed by 10 mg/kg per day in drinking water for 5 d per week for another 8 weeks.

After 24 weeks, the surviving animals were sacrificed using 200 g/L ethyl carbamate as anesthesia. The body, liver, spleen, and lungs were weighed, and an autopsy was performed. The liver nodules or tumors that were visible on the liver surface and the liver nodules which is greater than 3 mm in diameter were counted and measured. Multiple samples were taken from the tumors and from the apparently non-neoplastic liver portions.

Animal grouping and treatment

The animals were divided into four groups (n = 15): the normal control group, the model control group, the 6 g/kg CE-SB treatment group, and the 12 g/kg CE-SB treatment group. Cancer was induced using the same protocol as describe before. In addition, CE-SB was co-administered at a daily oral dose of 6 g/kg or 12 g/kg per day for 5 d per week for the total experimental period.

Histopathological examination of liver tissue

Hepatic tissues were fixed in a 40 g/L formaldehyde solution in 0.1 mol/L phosphate-buffered saline (pH 7.4), and embedded in paraffin. Section slides (5-6 μm thick) were prepared and stained with hematoxylin and eosin (HE).

Each liver was microscopically examined for lesion distributions, and were classified as focal, multifocal, diffused, and no detected abnormalities. The patterns of liver injury and lesion grading, which were classified as minimal, moderate, and marked, were determined in the coded slides. The liver sections were also observed for any necrosis/abscesses, degenerative changes, structural disruption, and any other changes.

Biochemical estimations

The separated plasma was analyzed for total protein and albumin content. The separated serum was analyzed for AST and ALT activities using a kit provided by Bicon, Germany. ALP was analyzed using a kit provided by Biolabo, France. Levels of MDA were determined as described by Yagi (1976). Activity of SOD was measured using commercial available kit purchased from Nanjing Jiancheng BioEngineering (Nanjing, China).

A 10% homogenate was obtained in a Tris-sucrose buffer (pH 7.4), and was centrifuged at 105,000 ×g at 4 °C for 30 min using a Dupont Sorvall Ultracentrifuge (USA) to isolate the cytosolic fraction, which was used for the assay of γ-Glutamyl transferase (γ-GT), glutathione-S-transferase (GST) and Alpha-L-fucosidase (AFU) activities.

Statistical analysis

All values were expressed as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) test and student t test using the statistical software SPSS 13.0. $P<0.05$ was considered statistically significant.

Results

Protective effects of CE-SB on DENA-Induced liver tumorigenesis

The rats in normal control group were active (eating and drinking). As shown in Table 1, the body weight in the CE-SB groups and the model group were significantly
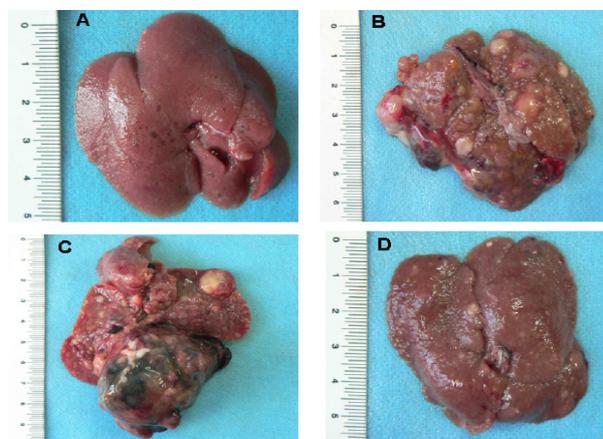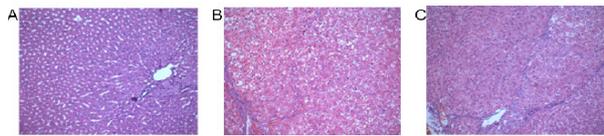

Figure 1. Liver Tissues of the Different Groups. (A) Liver tissue of the normal control group showed smooth surfaces, sharp edges, medium texture, and were dark red; (B) Liver tissue of the model control group rats showed multiple gray-white nodules of varying sizes; (C) Liver tissue of the model control group showed huge tumor formation; (D) Liver tissue of the 6 g/kg CE-SB group showed multiple liver surface nodules, but the nodules were significantly reduced compared with the model control group
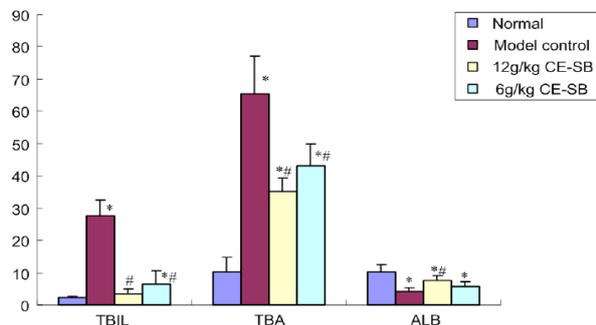
Table 1. Protective Effects of CE-SB on DENA-Induced Liver Tumorigenesis (±s)

Group	Dosage (g/kg/d)	Liver nodules	Body weight (g)	Liver weight (g)	Liver / body weight ratio (%)	Mortality rate (%)
Normal	-	0	496.53±63.45	11.62±2.34	2.34	0 (0/15)
Model control	-	40.22±4.83	343.62±76.52*	18.72±8.63*	5.45*	40.0(6/15)
CE-SB	12	13.33±2.12#	418.27±59.64*	14.73±3.26*	3.52*#	6.7(1/15)#
	6	24.54±3.05#	425.46±54.32*#	16.28±3.57*#	3.83*#	20.0(3/15)#

*P<0.05 versus normal group; # P<0.05 versus model control group

Figure 2. Liver Sections of the Different Groups. (A) Liver tissue of the normal control group showed hepatic lobule having normal architecture. The liver cell cord was arranged in neat rows, and a clear nucleus was observed (HE×200); (B) Liver tissue of the model control group showed nuclear pleomorphism. The lobular architecture of the model control group was normal, and the general concept of the white nodules of hepatocellular carcinoma cells were destroyed with low differentiation degree, significant atypical, and were mostly level III histological grade (HE×200); (C) CE-SB-treated rat liver tissues showed some degenerative changes and a vacuolated cytoplasm with high cell differentiation and were mostly level I-II histological grade (HE×200)
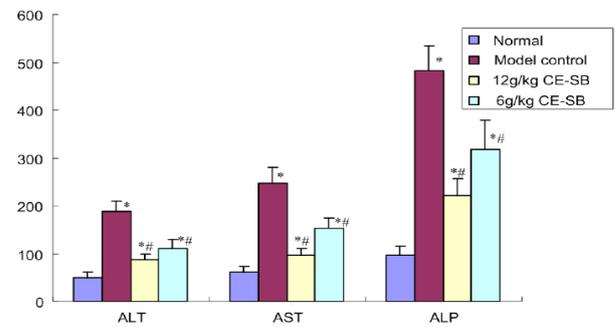

Figure 4. Effect of CE-SB on Enzymatic Liver Functions in DENA-induced Liver Tumorigenesis. Values are expressed as means ± standard deviations, and determined using the ANOVA test. *$P<0.05$ versus normal group, #$P<0.05$ versus model control group

Figure 3. Effects of CE-SB on Non-enzymatic Liver Functions in DENA-induced Liver Tumorigenesis. Values are expressed as means ± standard deviations, and determined using the ANOVA test. *$P<0.05$ versus normal group, #$P<0.05$ versus model control group

Figure 5. Effect of CE-SB on Tumor Markers in DENA-induced Liver Tumorigenesis. Values are expressed as means ± standard deviations, and determined using the ANOVA test. *$P<0.05$ versus normal group, #$P<0.05$ versus model control group

lower than the normal group ($P< 0.05$). The liver weight and the liver/body weight ratio in the CE-SB groups were significantly higher than the normal group, whereas liver nodules, liver weight, and liver/body weight ratio were significantly lower than the model group. However, the body weight of rats in the CE-SB groups were significantly higher than the model group, whereas the liver nodules and liver/body weight ratio were significantly lower than the model group (0.05). No significant difference was observed between the two CE-SB groups ($P >0.05$).
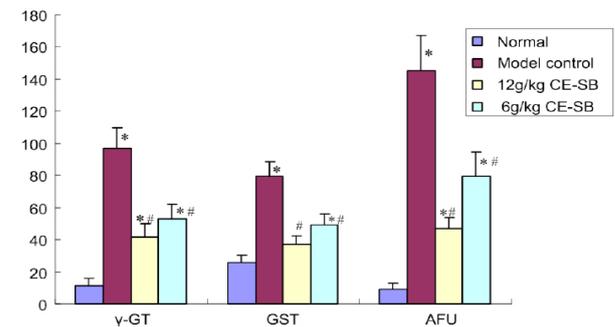
6 rats were died in the model group, 3 in the 6 g/kg CE-SB group, and 1 in the 12 g/kg CE-SB group. The mortality rate (%) in the model group, 6 g/kg CE-SB group and 12 g/kg CE-SB group were 40.0%, 20.0% and 6.7%, respectively.

Histopathological observation

The liver tissues of the normal control group were dark red with smooth surface, sharp edges, and medium texture (Figure 2A). The rats in the model control group and CE-SB groups were all successively induced with liver cancer. Multiple gray-white nodules of varying sizes were scattered in the liver tissues of the model control group (Figure 2B), of which four cases had huge tumor formation with a diameter greater than 3 cm (Figure 2C). The liver tissues of two cases in the 12 g/kg CE-SB group showed smooth surface and normal texture, but only a small focal nodule formation. The liver tissues of the 6 g/

kg CE-SB group showed multiple liver surface nodules, but the nodules were significantly reduced compared with the model control group (Figure 2D).

No case with spontaneous hepatocellular carcinoma was found by HE staining in the normal control group. The normal control group showed complete hepatic lobule structure. Moreover, the liver cell cord arranged in neat rows, and a clear nucleus (Figure 3A). The normal lobular architecture of the model control group and the general concept of the white nodules of hepatocellular carcinoma cells were destroyed with a low differentiation degree, significant atypical, and were mostly level III histological grade (Figure 3B). The microscopy results showed high cell differentiation in the liver cancer cells in the ESB groups and mostly level I-II histological grade (Figure 3C).

Effect of CE-SB on Non-enzymatic Liver Functions in DENA-Induced Liver Tumorigenesis

DENA significantly decreased total protein, albumin, and A/G levels, which indicated poor liver function and inability to fight infections. On the contrary, the administration of CE-SB normalized total protein, albumin, and A/G levels. It indicated that CE-SB could ameliorate liver function in DENA-induced liver damage DENA significantly decreased ALB content. The co-administration of CE-SB increased total protein, albumin, and A/G ratios. Moreover, DENA significantly increased total bilirubin (TBIL) and total bile acid (TBA) contents compared with the normal control group. CE-SB
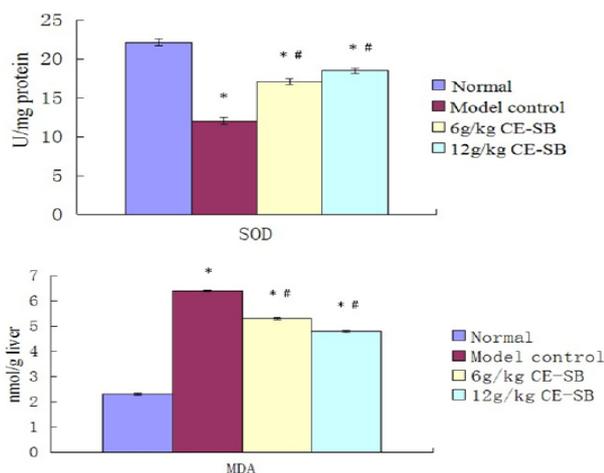
Figure 6. Effect of CE-SB on Oxidative Stress in DENA-induced Liver Tumorigenesis. Values are expressed as means ± standard deviations, and determined using the student's t-test test. *P<0.05 versus normal group, #P<0.05 versus model control group

significantly decreased TBIL and TBA contents compared with the model control group (Figure 3).

Effect of CE-SB on Enzymatic Liver Functions in DENA Induced Liver Tumorigenesis

DENA significantly elevated serum ALT, AST, and ALP activities compared with the normal group (Figure 4). The co-administration of CE-SB restored serum AST and GGT activities with significantly decreased ALP activity compared with the model control group (*P*<0.05).

Effect of CE-SB on Tumor Markers in DENA- Induced Liver Tumorigenesis

As shown in Figure 5, serum γ-GT, GST, and AFU activities were significantly elevated in the model control group compared with the normal group. The co-administration of CE-SB decreased the activities of serum tumor markers in rats with DENA-induced liver cancer, which were significantly different from the model control group (*P*<0.05).

Effect of CE-SB on Oxidative Stress in DENA-induced Liver Tumorigenesis

MDA and SOD values are shown in Figure 6. The MDA levels were significantly increased and SOD actives were reduced in rats with DENA-induced liver cancer, which is inhibite by CE-SB. It indicated that CE-SB may be useful to ameliorate liver injury caused by DENA-induced liver tumorigenesis.

Discussion

In this study, CE-SB (12 or 6 g/kg) was demonstrated to significantly reduce the numbers of the tumor nodules, improve the serum biochemical indices, and inhibit the levels of tumor markers induced by DENA. CE-SB treatment also significantly inhibited the DENA-induced liver weight increases and improved the hepatic histopathological damage induced by DENA. The numbers of hyperplastic nodules are correlate with hepatocarcinoma in experimental and human disease

(Bishayee et al., 1995). Therefore, the inhibition of nodule growth by CE-SB in our experiment was important for hepatocarcinoma prevention.

Low albumin is a sign of poor health and is a predictor of bad outcomes. Thus, a decreased A/G ratio often indicates the presence of impaired liver function (Ibrahim et al., 2008). DENA significantly decreased total protein, albumin, and A/G levels, which indicated poor liver function and inability to fight infections. On the contrary, the administration of CE-SB normalized total protein, albumin, and A/G levels, which indicated the ability of CE-SB to improve liver function in DENA-induced liver damage.

Serum AST, ALP, and GGT are sensitive hepatic injury indicators. AST and ALT activities increased during DENA-induced liver tumorigenesis (Dakshayani et al., 2005). The elevated activities of serum AST, ALP, and GGT that were observed in DENA-treated rats are due to the DENA-induced hepatic damage and the subsequent enzyme leakage into the circulation (Bansal et al., 2005). The administration of CE-SB restored the enzyme activities to nearly normal values, which indicated a hepatoprotective role of CE-SB.

γ-GT in healthy adults is mainly secreted by hepatic Kupffer cells and endothelial cells of the bile duct with obvious increased activity in HCC and fetal liver tissues (Cui et al., 2003). Serum γ-GT activity is one of the best liver damage indicators (Bulle et al., 1990). Serum GST is a class of enzymes involved in the detoxification function of the body by DENA and other chemical carcinogens. The increased GST level is likely to be the key drug resistance mediator in cancer chemotherapy (Rajkapoor et al., 2005). AFU is an enzyme that hydrolyzes fucose glycosidic linkages of glycoproteins and glycolipids (Zhou et al., 2006). AFU is a good tumor marker in early HCC detection (Ishizuka et al., 2007). γ-GT, GST, and AFU in the model group and CE-SB treatment groups increased in varying degrees. Tumor marker restoration was observed in the CE-SB treatment groups, which shows the protective effect of the CE-SB.

Liver injury could induce the oxidative stress by generation of reactive oxygen species (ROS), while SOD, the antioxidant enzymes against oxidative damage, could clear away ROS (Manoharan et al., 2012). MDA is a prouuct of polyunsaturated fatty acids exposed to ROS. In this study, we found the rats treated with DENA showed a increased MDA levels and an reduced SOD actives, while CE-SB could inhibites those changes. CE-SB may ameliorate the liver injury induced of DENE by antioxidant effect.

In conclusion, the protective effects of CE-SB crude extract against rat liver tumorigenesis were observed. The levels of liver function indexes in the CE-SB groups were lower than that of the model group. The results of the histological examination demonstrated that the number of liver cancer nodus in the CE-SB groups decreased compared with the model group. CE-SB can inhibit experimental liver tumorigenesis and can relieve hepatic injury in rats. However, further studies are necessary to clarify the detailed mechanism involved in the antitumor effects of CE-SB.

Acknowledgements

References

Bansal AK, Bansal M, Soni G, et al (2005). Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem Biol Interact*, **156**, 101-11.

Bhosale P, Motiwale L, Ingle AD, et al (2002). Protective effect of Rhodotorula glutinis NCIM3353 on the development of hepatic preneoplastic lesions. *Curr. Sci*, **83**, 303-8.

Bishayee A, Chatterjee M (1995). Inhibitory effect of vanadium on rat liver carcinogenesis initiated with diethylnitrosamine and promoted by phenobarbital. *Br Cancer J*, **71**, 1214-20

Bulle F, Mavier P, Zafrani ES, et al (1990). Mechanism of gamma-glutamyl transpeptidase release in serum during intrahepatic and extrahepatic cholestasis in the rat: a histochemical, biochemical and molecular approach. *Hepatology*, **11**, 545-50.

Cui R, He J, Zhang F, et al (2003). Diagnostic value of protein induced by vitamin K absence(PIVKAII) and hepatoma-specific band of serum gamma-glutamyl transferase (GGTII) as hepatocellular carcinoma markers complementary to alpha-fetoprotein. *Br J Cancer*, **88**, 1878-82.

Dai SJ, Peng WB, Shen L, et al (2011). New norditerpenoid alkaloids from Scutellaria barbata with cytotoxic activities. *Nat*. *Prod*. *Res*, **15**, 1-6.

Dai SJ, Sun JY, Ren Y, et al (2007). Bioactive ent-clerodane diterpenoids from Scutellaria barbata. *Planta Med*, **73**, 1217-20.

Dai ZJ, Gao J, Li ZF, et al (2011). In Vitro and In Vivo Antitumor Activity of Scutellaria Barbate Extract on Murine Liver Cancer. *Molecules*, **16**, 4389-400.

Dai ZJ, Wang XJ, Li ZF, et al (2008). Scutellaria barbate extract induces apoptosis of hepatoma H22 cells via the mitochondrial pathway involving caspase-3. *World J Gastroenterol*, **14**, 7321-8.

Dakshayani KB, Subramanian P, Manivasagam T, et al (2005). Melatonin modulates the oxidantantioxidant imbalance during N- nitrosodiethy- lamine induced hepatocarcinogenesis in rats. *J Pharm Pharm Sci*, **8**, 316-21.

Farazi1 PA, DePinho RA (2006). Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer*, **6**, 674-87.

Futakuchi M, Hirose M, Ogiso T, et al (1999). Establishment of an in vivo highly metastatic rat hepato-cellular carcinoma model. Jpn J Cancer Res, 90, 1196-202.

Goh D, Lee YH, Ong ES (2005). Inhibitory effects of a chemically standardized extract from Scutellaria barbata in human colon cancer cell lines, LoVo. *J Agric Food Chem*, **53**, 8197-204.

Ibrahim SS, Nassar NN (2008). Diallyl sulfide protects against N-nitrosodiethylamine induced liver tumorigenesis: Role of aldose reductase. *World J Gastroenterol*, **14**, 6145-53.

Ishizuka H, Nakayama T, Matsuoka S, et al (1999). Prediction of the development of hepato-cellular-carcinoma in patients with liver cirrhosis by the serial determinations of serum alpha-L-fucosidase activity. *Intern Med*, **38**, 927-31.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Lee GH, Ooasa T, Osanai M (1998). Mechanism of the paradoxical, inhibitory effect of phenobarbital on hepatocarcinogenesis initiated in preweaning B6C3F1 mice with diethylnitrosamine. *Cancer Res*, **58**, 1665-9.

Lee TK, Lee DK, Kim DI (2004). Inhibitory effects of Scutellaria barbata D.Don on human uterine leiomyomal smooth muscle cell proliferation through cell cycle analysis. *Int Immunopharmacol*, **4**, 447-54.

Lin CC, Shieh DE (1996). The anti-inflammatory activity of Scutellaria rivularis extracts and its active components, baicalin, baicaleinand wogonin. *Am J Chin Med*, **24**, 31-36.

Manoharan S, Singh AK, Suresh K, et al (2012). Anti-tumor Initiating Potential of Andrographolide in 7,12-dimethylbenz[a]anthracene Induced Hamster Buccal Pouch Carcinogenesis. *Asian Pac J Cancer Prev*, **13**, 5701-8.

Mi X, Zhu R (2010). Simultaneous determination of 7 active ingredients in Scutellaria barbata D. Don by capillary micellar electrokinetic chromatography. *Se Pu*, **28**, 209-214.

Qu GW, Yue XD, Li GS, et al (2010). Two new cytotoxic ent-clerodane diterpenoids from Scutellaria barbata. *J Asian Nat Prod Res*, 12, 859-64.

Rajkapoor B, Murugesh N, Chodon D, et al (2005). Chemoprevention of N-nitrosodiethylamine induced phenobarbitol promoted liver tumors in rat by extract of Indigofera aspalathoides. *Biol Pharm Bull*, **28**, 364-6.

Suh SJ, Yoon JW, Lee TK, et al (2007). Chemoprevention of Scutellaria bardata on Human Cancer Cells and Tumorigenesis in Skin Cancer. *Phytother Res*, **21**,135-41.

Vickers, A (2002). Botanical medicines for the treatment of cancer: Rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Invest*, **20**, 1069-79.

Yagi K (1976). A simple fluorometric assay for lipoperoxide inblood plasma. *Biochem Med*, **15**, 212-6.

Yin X, Zhou J, Jie C, et al (2004). Anticanceractivity and mechanism of Scutellaria barbata extract on human lung cancer cell line A549. *Life Sci*, **75**, 2233-44.

Zhou L, Liu J, Luo, F (2006). Serum tumor markers for detection of hepatocellular carcinoma. *World J Gastroenterol*, **12**, 1175-81.